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FIRST INTERNATIONAL SYMPOSIUM ON THE SYNOVIUM—PART 2

Immunohistologic Study of T-Cell Receptor δ-Chain Expression in Rheumatoid Synovial Membranes

By Hani S. El-Gabalawy and James Keillor

Lymphocytes expressing γδ T-cell receptors (TCRs) have been shown to be reactive to mycobacterial antigens as well as the so-called stress proteins. The detection of increased numbers of γδ cells in the synovial fluid and peripheral blood of some patients with rheumatoid arthritis has suggested a potential role for these lymphocytes in the pathogenesis of this disorder. Twenty-three rheumatoid synovial membranes were studied using immunohistology and monoclonal antibodies in an attempt to define the patterns of distribution of γδ T cells in rheumatoid synovitis. Consecutive sections were stained for T1(CD5), T4(CD4), T8(CD8), TAC(CD25), the δ-chain markers δTCR1 and δTCS1, and the β-chain marker βF1. Our results show some regional differences in the distribution of CD4 and CD8 cells, the former being prominent in the lymphocytic aggregates and the latter most prominent in diffuse infiltrates immediately adjacent to the synovial lining layer. All tissues showed extensive staining for βF1; an estimated average of more than 90% of T cells expressed αβ TCR. The majority of samples showed limited staining for both δ-chain antibodies, with 20 of the 23 tissues appearing to have less than 1% of T lymphocytes expressing these markers. Three tissues stained extensively for both δTCR1 and δTCS1 in particular areas of the section. In these areas, small perivascular lymphocytic aggregates appeared to be composed mainly of γδ cells. TAC staining was virtually absent in all areas and tissues. It was concluded that the majority of T lymphocytes infiltrating rheumatoid synovial membranes express αβ TCR. An occasional tissue will show an area of intense γδ-cell infiltration, the distribution of which suggests some form of clonal expansion. These areas may be the site of unique immunopathologic processes.

INDEX WORDS: γδ T-cell receptor; rheumatoid arthritis; immunohistochemistry; synovium.

WHEREAS MOST peripheral blood $CD3^+$ T lymphocytes express an αβ heterodimer as an antigen receptor (T-cell receptor [TCR]), an average of 5% express γδ receptors encoded by a unique set of genes.[1-3] For the most part, these γδ T cells express a $CD3^+/CD4^-/CD8^-$ phenotype.[4,5] During thymic development there appears to be earlier rearrangement of the γ and δ genes as compared with the α and β genes, resulting in the expression of γ δ receptors before αβ receptors.[6-9] Outside the thymus, γδ cells are prominant in epithelial layers of the skin and the intestine.[10,11] In these areas, a striking compartmentalization is evident in the utilization of variable region genes (Vγ and Vδ) by these cells.[12] It is uncertain whether the cells rearrange in the thymus and home to the periphery or whether there is clonal selection in the peripheral microenvironment.

The particular responsiveness of these T cells toward mycobacterial antigens has been shown in studies in both humans and mice.[13-15] The fact that at least 10% of γδ-bearing hybridomas derived from neonatal mouse thymocytes were reactive to mycobacteria is quite remarkable, particularly considering that the animals were unprimed.[14] More than half of these purified protein derivative (PPD)-reactive hybridomas also reacted to mycobacterial heat-shock protein (HSP) 65 and the majority secreted interleukin-2 (IL-2) spontaneously in the absence of any added antigen. The particular reactivity of γδ

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cells toward stress proteins has been well documented (reviewed by Born et al[16]).

Attention has focused on the role of mycobacterial antigens and stress proteins in the pathogenesis of chronic inflammatory arthropathies such as rheumatoid arthritis (RA).[17] The ability of mycobacterial antigens to induce adjuvant arthritis is well established and appears to relate, at least in part, to immunodominant epitopes on the mycobacterial 65K protein.[18] In RA the levels of antibodies to mycobacterial HSP65 are elevated.[19] More importantly, rheumatoid synovial fluid T cells show reactivity to mycobacterial HSP65,[13,20,21] while this protein is abundantly evident in inflamed synovial tissues.[22] Taken together, these observations suggest that immune responses toward stress proteins are potentially relevent in the pathogenesis of RA.

Some patients with RA have increased numbers of lymphocytes expressing γδ receptors in the synovial fluid and blood when compared with normal controls.[23-25] In certain individuals, the levels were as high as 25% in the synovial fluids. This increased ratio of γδ cells appeared to be a consistent finding when more than one joint was studied in the same individual.[25] γδ cells cloned from the synovial fluid of a rheumatoid patient were all responsive to mycobacterial antigens, and one was responsive to the HSP65.[13] The gene utilization patterns of the rheumatoid synovial γδ cells appears to differ from those derived from the peripheral blood; the former utilizes predominantly Vδ1 and the latter Vδ2.[26] This further attests to the compartmentalization of these cells.

Several monoclonal antibodies specific for δ-chain epitopes have been developed and studied in detail. The δTCR1 antibody has been shown to be specific for a common epitope on the δ chain that is present on essentially all peripheral γδ cells.[3] δTCS1 recognizes a variable region epitope[27] which is a Vδ1-Jδ1 gene product[28] and is detected on a fraction of γδ cells in peripheral blood.[3]

To clarify further the potential role of γδ cells in rheumatoid synovitis, we studied the distribution of cells staining for the δ chain of the TCR in rheumatoid synovial membranes using the monoclonal antibodies δTCR1 and δTCS1. We compared this with the distribution of T-cell subsets and the β-chain marker βF1 in consecutive sections of the same tissue.

METHODS AND MATERIALS

Twenty-three rheumatoid synovial membranes obtained from patients with definite RA at the time of synovectomy or arthroplasty were snap frozen using liquid nitrogen and stored at −70°C for subsequent staining.

An avidin-biotin immunoperoxidase technique was used to stain the tissues. The monoclonal antibodies used included the DAKO products T1 (anti-CD5), T4 (anti-CD4), T8 (anti-CD8), and TAC (anti-CD25) (DAKO Corporation, Carpinteria, CA). The βF1 (β chain of TCR), δTCR1 (δ chain of TCR, common epitope), and δTCS1 (δ chain of TCR, variable epitope) antibodies were obtained from T Cell Sciences (Cambridge, MA). Briefly, cryostat sections were acetone fixed and air dried. These primary antibodies were applied in the dilutions recommended by the manufacturer, incubated for 30 minutes, and washed with Tris buffer. The cryostat sections were then biotinylated for 30 minutes with rabbit anti-mouse antibody and the avidin-biotin complex, then washed again with Tris buffer. The slides were developed in diaminobenzidine and counterstained with hematoxylin, then dehydrated and mounted.

Consecutive sections were used to follow the relative distribution of the markers in particular areas. A scale of 0 to 3 was used to describe the degree of staining: 0, none; 1, sparse (accounting for $<10\%$ of the total mononuclear cell infiltrate in the field); 2, moderate (10% to 50%); 3, intense (50% to 100%). In addition, manual counts of positively staining cells in five representative fields of each section were obtained. Staining for the T1 antibody was used as a reference for estimating the relative percentage of T cell expressing the TCR-chain markers.

Where applicable, statistical significance was assessed using the Mann-Whitney test.

RESULTS

All rheumatoid synovial tissues studied showed the presence of T lymphocytes. The most common pattern of infiltration was perivascular lymphocytic aggregates of various sizes. Five patients (22%) showed only a diffuse

infiltrate of lymphocytes that tended to favor areas immediately adjacent to the synovial lining layer. Table 1 summarizes the average intensity of immunoperoxidase staining (0 to 3+) detected in these two types of infiltrates using our panel of monoclonal antibodies. The lymphocytic aggregates, particularly the larger collections, consistently had a predominance of $CD4^+$ compared with $CD8^+$ cells. On the other hand, the density of $CD8^+$ cells tended to be highest in the more diffuse infiltrates adjacent to the synovial lining layer (Fig 1B). The average CD4-CD8 ratio in the latter areas was 0.78, compared with 1.51 in the nodular lymphocytic aggregates ($P < .001$). There were no obvious differences in the distribution of αβ TCR, γδ TCR, or CD25 between the two types of infiltrates.

Staining patterns for βF1 and T1 were quantitatively very similar, with more than 90% of T cells in the tissues appearing to express αβ receptors. In most of the tissues, very little δ-chain staining was evident using either δTCR1 or δTCS1. A few isolated cells stained for these antibodies (Fig 1C), and overall they accounted for less than 1% of the T-cell infiltrate. There were only minor quantitative differences between the degree of δTCR1 and δTCS1 staining, the former consistently staining a few more cells.

Three of the 23 tissues showed an area characterized by small aggregates of lymphocytes, a high percentage of which were stained by both of the δ-chain antibodies (Fig 2). In some of the aggregates, more than 50% of the total T-cell infiltrate appeared to express the δ-chain markers. Compared with the average number of δ-chain–positive cells in the tissues as a whole, the differences were highly significant (85 *v* 6 per high-power field; $P < .0001$). These areas were unique to one part of the tissue section and were shown in sequential sections. Adjacent areas equally infiltrated with lymphocytes showed a virtually complete absence of δ-chain staining (Fig 2). The clinical characteristics of the three patients did not differ from those of the group as a whole (data not shown).

The expression of TAC (CD25) was limited in the tissues; only the so-called transitional areas at the periphery of the larger lymphocytic aggregates showed occasional staining.

Table 1: Intensity of Immunoperoxidase Staining Evident in Two Types of Infiltrates in Rheumatoid Synovial Membranes: Diffuse Lymphocytic Infiltrates and Nodular Lymphocytic Aggregates

Antibody	Diffuse Infiltrates*	Nodular Aggregates*
T1	+++	+++
T4	++	+++
T8	+++	++
βF1	+++	+++
δTCR1	0/+	0/+
δTCS1	0/+	0/+
TAC	0	0/+

*0, no staining; +, 1% to 10%; ++, 10% to 50%; +++, 50% to 100% of total T cells (as defined by the T1 antibody).

DISCUSSION

Studies of lymphocyte populations in rheumatoid joints have generally shown a tendency for $CD4^+$ T cells to aggregate in the synovial membrane while $CD8^+$ T cells are prominant in the synovial fluid (reviewed by Firestein et al[29]). Our findings show that in contrast to the high CD4-CD8 ratios in the lymphocytic aggregates, the immediate sublining areas closest to the joint cavity consistently had lower CD4-CD8 ratios, with $CD8^+$ cells often predominating. This may help explain the low CD4-CD8 ratio generally detected in rheumatoid synovial fluids,[30,31] which may reflect the ratio in the superficial areas of the membrane.

Our immunohistochemical observations suggest that the majority of rheumatoid synovial T lymphocytes expressed αβ receptors. The number of lymphocytes expressing the δ chain appeared to be less than 1% in most tissues. This is in contrast to an average of 15% in the synovial fluids of the rheumatoid patients studied by Rème et al in their flow-cytometric study[25] using the TiγA antibody, which recognizes the Vγ9 subset of γδ T cells. It is unlikely that we did not detect all the γδ cells in the tissue sections because, as stated above, the δTCR1 antibody appears to define an epitope that is present on essentially all of this T-cell subset.[3] Recent studies in two other laboratories have not shown a significant degree of γδ T-cell sequestration in the joints of adults with rheumatoid arthritis.[32,33]

Both of these studies also confirmed that the majority of the γδ cells present in the joints of patients with RA and juvenile RA appear to be utilizing the Vδ1 gene. The detection of only minor quantitative differences between δTCR1 and δTCS1 staining in our study tissues is consistent with these observations. Furthermore, a high proportion of the synovial cells responding to mycobacterial HSP65 were TCS-1+ γδ cells.[34] Interestingly, none of the four γδ cell clones isolated by Holoshitz et al from a rheumatoid joint were recognized by δTCS1, although they were positive for δTCR1.[13]

It is possible that γδ T cells play a significant role only in certain subsets of patients with inflammatory arthropathies. This appeared to be the case in a cohort of juvenile RA patients studied by Kjeldsen-Kragh et al.[32] In addition, this subset of lymphocytes may be more important in the early pathogenetic mechanisms of inflammatory arthropathies than when the disorder becomes more established. In the study of

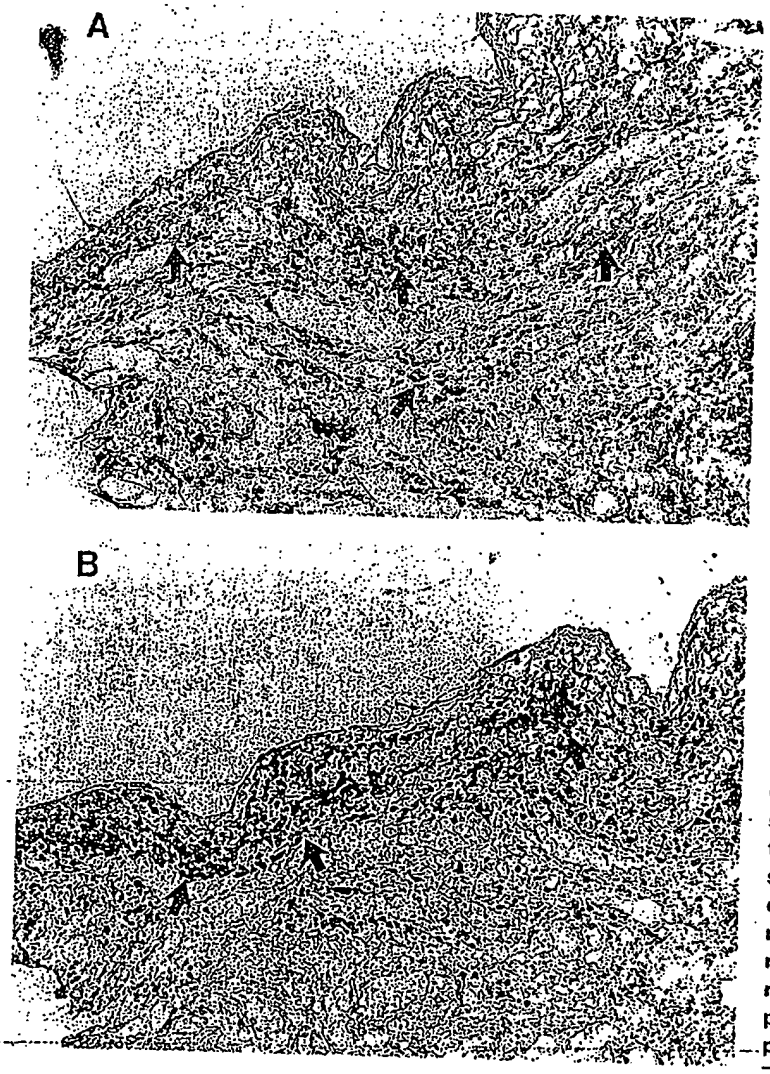

Fig 1: Low-power views (original magnification ×40) of sequential sections of a rheumatoid synovial membrane stained using monoclonal antibodies and an immunoperoxidase technique. (A) T4 (anti-CD4) staining is diffuse and widespread (arrows). (B) T8 (anti-CD8) staining is most dense in the areas adjacent to the synovial lining layer, closest to the joint cavity (arrows). This results in a relatively low CD4-CD8 ratio in these areas compared with nodular lymphocytic aggregates.

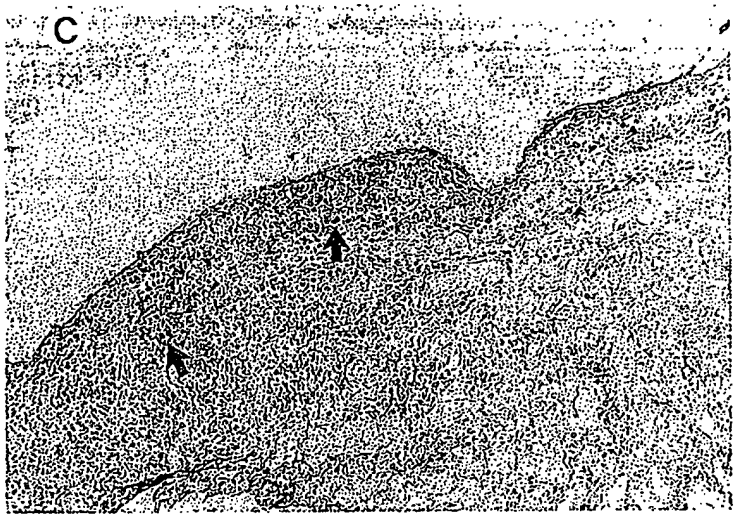

Fig 1 (cont'd): (C) Section stained with δTCR1 showing a sparse infiltration of γδ cells (arrows).

Brennan et al, one patient with new-onset disease showed high levels in both blood and synovial fluid.[23] The heightened reactivity of synovial T cell to the HSP65 evident in early RA is also in keeping with this concept.[21] Our immunohistochemical study was conducted using samples from patients tending to have more advanced synovial lesions. It is possible that tissues obtained earlier in the course of RA would show more abundant infiltration with γδ T cells.

The most striking observation in this study relates to the detection of one area in each of three tissues that showed a marked increase in the number of cells expressing δ chains. We were surprised to see γδ-rich lymphocytic aggregates adjacent to aggregates that were virtually devoid of any cells expressing the δ-chain markers (Fig 2). The mechanisms responsible for such an observation could involve either a preferential recruitment of γδ cells or in situ clonal expansion of these cells. Assessment of

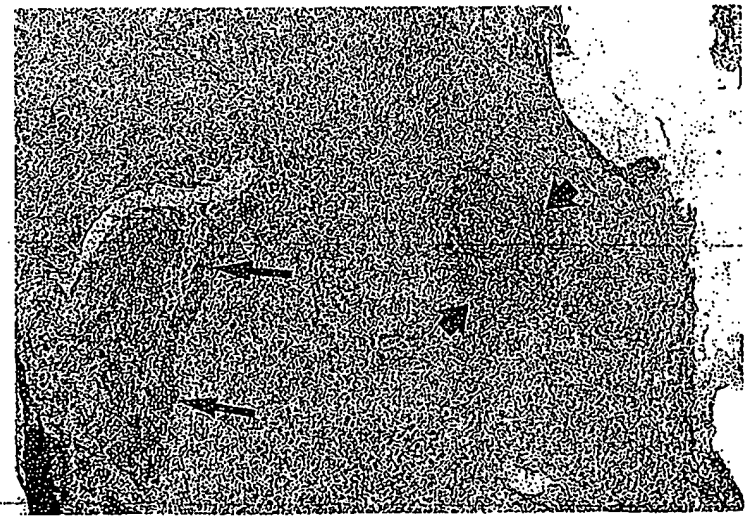

Fig 2: Low-power view (original magnification ×40) of two adjacent nodular lymphocytic aggregates in rheumatoid synovial tissue. One aggregate is rich in δ-chain staining (δTCS1) (arrows); the other is almost devoid of cells staining for this marker (arrowheads). This appearance is suggestive of in situ clonal expansion of γδ cells.

clonal dominance in rheumatoid T cells by restriction fragment–length polymorphism methodology has generated conflicting results.[35,36] Further experimental evidence is necessary to clarify whether accumulations of γδ cells as detected in some of our tissues are indeed clonally restricted.

In summary, we have shown that the overwhelming majority of lymphocytes in rheumatoid synovial membranes express αβ TCR. The tissues studied had evidence of small numbers of single cells expressing γδ TCR, which appeared to be distributed randomly. In a minority of tissues, isolated areas of high γδ T-cell density are evident; the significance of this finding is unclear.

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